Induction of Influenza A Virus Cross-reactive Cytotoxic T Cells by a Nucleoprotein/Haemagglutinin Preparation

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(Accepted 1 March 1985)

SUMMARY

An ammonium deoxycholate fraction from bromelain-treated influenza A virus was highly enriched for virus nucleoprotein and contained residual haemagglutinin (NP/HA). The preparation did not contain detectable levels of matrix or neuraminidase proteins and was free of infectious virus. NP/HA effectively primed mice for cytotoxic T cells which lysed syngeneic cells infected with any type A influenza virus. Furthermore, NP/HA generated A-type virus cross-reactive cytotoxic T cells when added in vitro to spleen cells from mice previously primed with infectious influenza A virus. These properties imply that NP/HA has potential as a vaccine for heterotypic influenza A immunity.

Cytotoxic T (Tc) cells play an important role in the immune response to and protection against viral infections. Anti-influenza Tc cells can protect unexposed mice from subsequent infection with influenza virus (Yap et al., 1978; Lin & Askonas, 1981; Lukacher et al., 1984). Most significantly, the majority of anti-influenza Tc cells have broad specificity within a particular influenza type (Zweerink et al., 1977b). This contrasts their recognition pattern with the antibody response which is largely influenza subtype-specific. Current influenza vaccines, while effectively stimulating subtype-specific antibody, induce insignificant Tc cell production (Webster & Askonas, 1980; Reiss & Schulman, 1980; P. M. Taylor & B. A. Askonas, unpublished observations).

It has recently been shown that recognition of infected cells by some cloned mouse Tc cells maps to the virus nucleoprotein (NP) gene (Townsend & Skehek 1982). Furthermore, influenza A virus cross-reactive Tc cells from C3H/He (H-2k) mice can lyse syngeneic L cells expressing cloned influenza A virus nucleoprotein (Townsend et al., 1984). These experiments imply that the viral NP could be immunogenic for cross-reactive Tc cells and should therefore be considered for vaccination purposes.

We have used a simple method for preparing influenza A virus NP to test its efficacy in stimulating cross-reactive anti-influenza Tc cells in mice. HAM, a mutant of A/X31 virus adapted to infect cells in the presence of horse serum, was treated with bromelain to release the majority of the surface glycoproteins (Brand & Skehel, 1972). This process was repeated a further three times and the viral cores were stored at 4 °C. Cores were resuspended to the equivalent of 1 x 10^6 haemagglutinating units (HAU) per ml of whole virus and treated with ammonium deoxycholate (Am.DOC) (0.2%, w/v) as described in detail elsewhere (Laver & Webster, 1976). After 5 h at room temperature, the sample was centrifuged at 10000 g for 30 min.

The supernatant and pellet were analysed by SDS–polyacrylamide gel electrophoresis on 5 to 20% gradient gels under non-reducing conditions (Laemmli, 1970). Coomassie Brilliant Blue staining showed that the 10000 g supernatant was highly enriched for NP with residual haemagglutinin (HA) (Fig. 1b). Parallel immunoblotting showed that this sample contained undetectable levels of neuraminidase (NA) and identified the higher molecular weight bands as...
Fig. 1. SDS-PAGE of Am.DOC-treated influenza virus fractions. (a) Standards: bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin in order of decreasing molecular weight. Relative molecular weight of these non-reduced proteins was calculated relative to the mobility of reduced proteins electrophoresed on the same gel. (b) NP/HA fraction: supernatant from Am.DOC-extracted virus centrifuged at 10000 g. (c) M fraction: pellet from Am.DOC-extracted virus resuspended in Sarkosyl (1%, w/v). (d) As (c), but supernatant after centrifugation at 100000 g. Note that, under these electrophoretic conditions, NP was separated into two bands and that multimers of the HA were identified by immunoblotting using a monoclonal anti-HA antibody.
Fig. 2. The NP/HA fraction stimulates influenza A virus cross-reactive Tc in vitro. Tc cells were generated by culturing spleen cells from mice primed intranasally with A/X31 virus with either (a) 1 μg/ml NP/HA, (b) 10 μg/ml NP/HA, (c) A/X31 virus-infected cells or (d) in the absence of antigen for 5 days. P815 cells were infected with virus and labelled with $^{51}$Cr for 1 h, washed, incubated in RPMI/10 for 3 h, washed again and used as targets in 3 h cytotoxicity assays (Wraith et al., 1983). Percentage lysis was calculated as described previously (Zweerink et al., 1977a). K/T = killer to target cell ratio at 2 $\times$ 10$^4$ targets per well. ○, P815 cells infected with A/X31; △, P815 infected with A/USSR; □, P815 infected with B/Hong Kong. Background release of $^{51}$Cr was less than 10%.

To ensure that NP/HA did not contain infectious virus particles, aliquots (10 μg) were injected in triplicate into 11-day embryonated chicken eggs. These were incubated at 33 °C for 3 days and 100 μl of allantoic fluid was passaged in fresh 11-day eggs for a further 3 days. In control experiments, no HA activity was recovered from either passagge, indicating that the NP/HA fraction did not contain infectious virus particles.

The NP/HA fraction at 1 to 10 μg/ml was able to generate influenza A virus cross-reactive Tc cells in vitro (Fig. 2). Responder cells were spleen cells from BALB/c (H-2$^d$) mice, which had been primed intranasally with A/X31 virus. They were cultured alone, as control, alternatively with 1 or 10 μg/ml NP/HA fraction or with A/X31 virus-infected syngeneic spleen cells at 10$^5$ cells/ml as described by Zweerink et al. (1977a). After 5 days, Tc cell cytotoxicity was tested using P815 target cells (H-2$^d$) infected with either the homologous A/X31 (H3N2), the heterologous A/USSR/90/77 (H1N1) or B/Hong Kong/8/73 viruses (Zweerink et al., 1977a). The NP/HA-stimulated cells lysed both homologous and heterologous A type virus-infected targets similarly to (Fig. 2a, b) cells stimulated with infective virus (Fig. 2c). There was no significant lysis of influenza B virus-infected targets by these cultures and insignificant influenza A virus-specific lysis by control cultures of responder cells in the absence of antigen (Fig. 2d).
We wished to examine whether the NP/HA fraction could prime hosts *in vivo* for cross-reactive anti-influenza A virus Tc cells. Three-month-old BALB/c mice, bred under specific pathogen-free conditions, were immunized by intraperitoneal injection with 50 μg of NP/HA suspended in phosphate-buffered saline (pH 7.2) (PBS/A). Similarly mice were injected with either PBS/A alone or 50 μg of the M fraction from the same Am.DOC preparation. After 3 weeks, spleen cells from the NP/HA-, M- or control-immunized mice were re-stimulated *in vitro* at 1 × 10⁶ cells/ml in RPMI/10 by the addition of 2 × 10⁵ syngeneic spleen cells/ml infected with A/X31 virus (Zweerink et al., 1977a). After 5 days, T cell-mediated cytotoxicity was tested as above. The results (Fig. 3) clearly show that the NP/HA fraction effectively primed hosts for anti-influenza cytotoxic T memory cells, since no specific Tc cells were induced in control spleen cells under these conditions (Fig. 3a).

Interestingly, the Sarkosyl-solubilized M fraction did not prime mice. This is noteworthy considering that this fraction clearly contains significant levels of NP (Fig. 1). There are three possibilities: (i) the M protein could suppress Tc cell priming *in vivo*, (ii) this fraction could be depleted of another component for effective priming, or (iii) Sarkosyl extraction of the M fraction could have denatured the NP to such an extent as to prevent effective Tc cell priming.

We conclude from these experiments that the NP/HA fraction of Am.DOC-extracted influenza virus cores effectively primes mice for influenza A virus cross-reactive Tc cells and also re-stimulates cross-reactive memory Tc cells *in vitro*. Previous reports regarding the stimulation of Tc cell responses *in vivo* using either inactivated virus or viral glycoproteins are somewhat controversial. Thus, with inactivated virus two groups were unable to induce any response (Braciale & Yap, 1978; Reiss & Schulman, 1980), one noted a subtype-specific response only (Ennis et al., 1977) while another noted a very low transient cross-reactive response (Webster & Askonas, 1980). The differences could relate to the route of immunization which was different in each case. Convincing evidence shows that HA/NA does not prime *in vivo* for cross-reactive Tc cells. Thus, neither Triton X-100 HA/NA administered intraperitoneally (Reiss & Schulman, 1980) nor Am.DOC HA/NA administered intramuscularly (Webster & Askonas, 1980) stimulated any Tc cell response at all. Furthermore, purified HA from A/Jap/170/62 (H2N2) when used to re-stimulate memory Tc cells *in vitro* selected only subtype-specific Tc cells (Zweerink et al., 1977a; Braciale, 1979). More recently in extensive studies with HA of the H3 subtype, the virus used in these studies, we have been unable to stimulate cross-reactive Tc cells (D. C. Wraith, unpublished observations). Such evidence implies that it is the NP of the Am.DOC preparation that stimulates cross-reactive Tc cells. This being the case, one questions whether the residual haemagglutinin of NP/HA is required for...
stimulation. It is possible that while NP can alone be presented to and stimulate Tc precursor cells, the HA stimulates additional T helper cells in order to produce a response. Further purification of both NP and HA should establish this point.

For vaccination purposes, one would ultimately select a preparation effective in stimulating both humoral and cellular immune responses. We would expect the NP/HA fraction to stimulate antibody to both components. While it is accepted that NP can stimulate antibody production (Virelizier et al., 1979) the role of such an antibody in protection is not clear. Antibody to HA, on the other hand, can neutralize free virus particles, albeit only of the same subtype. Priming for anti-HA antibody is thus advantageous in unexposed hosts, such as young children, and would favour the inclusion of HA in a vaccine preparation whether or not it contributes to a Tc cell response.

As cross-protection by Tc cells has been described by three independent laboratories (Yap et al., 1978; Lin & Askonas, 1981; Lukacher et al., 1984), the cross-reactive Tc cell response stimulated by NP/HA is particularly encouraging. This preparation therefore has potential as an anti-influenza vaccine. Experiments to test for protection against lethal infection in mice are in progress.

We are most grateful to John Skehel for the gift of bromelain-treated purified HAM virus and to Angela Vessey for technical assistance.

REFERENCES


(Received 30 January 1985)